

**PURIFICATION, CHARACTERIZATION AND RECONSTITUTION
OF THE ATP SYNTHETASE FROM YEAST MITOCHONDRIA**

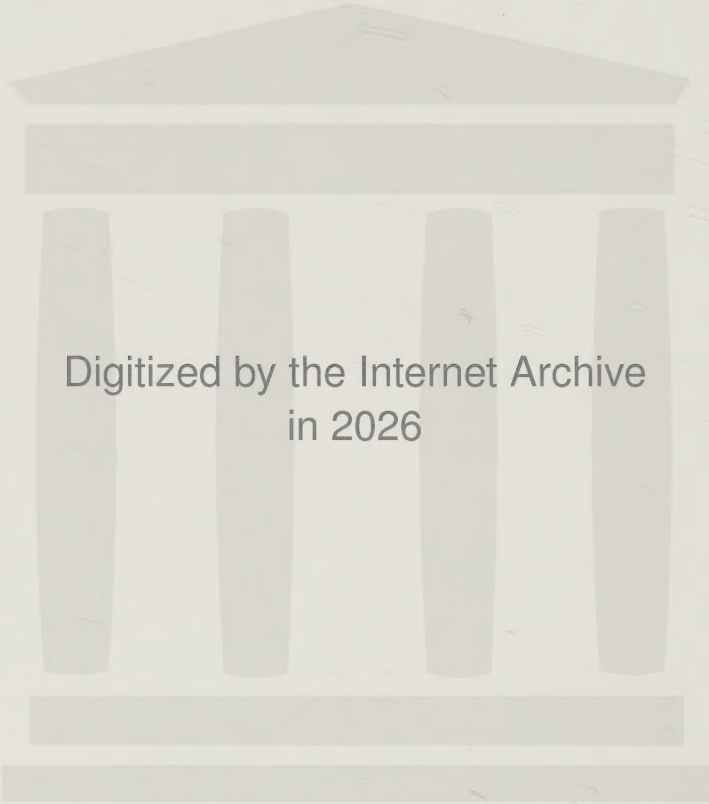
DISSERTATION

submitted for
the degree of Doctor of Philosophy
at the Department of Biology,
Ruhr-Universität Bochum

MA. YOLANDA GÓMEZ MAQUEO DE COVARRUBIAS

Bochum ~~1984~~

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The present work was done at the Max-Planck-Institut für
Ernährungsphysiologie, Dortmund. Director: Prof. Benno Hess.

REINIGUNG, CHARAKTERISIERUNG UND REKONSTITUTION
DER ATP SYNTHETASE AUS HEFE-MITOCHONDRIEN

D I S S E R T A T I O N

zur Erlangung des Grades
eines Doktors der Naturwissenschaften
der Abteilung für Biologie
an der Ruhr-Universität Bochum

von

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Bochum 1984

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ABBREVIATIONS used:

9-AA	9-aminoacridine
ACMA	9-amino-6-chloro-2-methoxyacridine
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
APS	ammonium persulfate
DCCD	N-N'-dicyclohexylcarbodiimide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
FCCP	carbonylcyanide-p-trifluoromethoxyphenyl-hydrazine
F_1	coupling factor 1 of the ATP synthetase
F_0	membrane sector of the ATP synthetase
F_6	coupling factor between F_1 and F_0
F_B	coupling factor B of the ATP synthetase
ATP synthetase	F_0 - F_1 complex
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HPLC	high pressure liquid chromatography
LDH	lactate dehydrogenase
MES	2- N-morpholino ethanol sulfonic acid
MW	molecular weight
NADH	reduced nicotinamide-adenine dinucleotide
OSCP	oligomycin sensitivity conferring protein
Oxonol VI	bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol
SDS	sodium dodecylsulfate
SMP	submitochondrial particles
TEMED	N,N,N',N',-tetramethylethylene-diamine
Tricine	N- tris(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)aminomethane
PEP	phosphoenolpyruvate
PK	pyruvate kinase

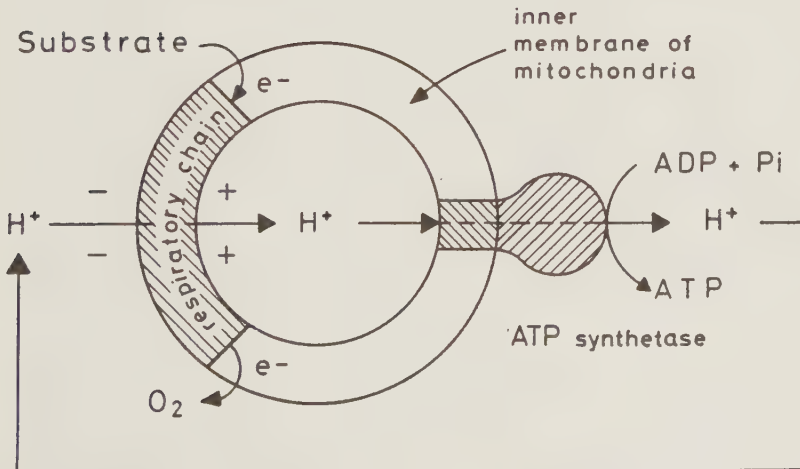
z	$2,3 RT/nF$
ΔpH	transmembrane pH gradient
$\Delta \psi$	transmembrane electrical potential
$\Delta \tilde{\mu}_{H^+}$	transmembrane electrochemical potential gradient

INTRODUCTION

The chemiosmotic theory (Mitchell, 1961, 1979) proposes that an electrochemical gradient of protons across the energy-transducing membranes of living organisms provides the energy necessary to synthesise ATP using ADP and P_i as substrates.

Since the respiratory chain of mitochondria pumps protons unidirectionally (i.e. vectorial transport (Jencks, 1983)), the asymmetrical distribution of protons ($Z\Delta pH$) together with the membrane potential ($\Delta\Psi$) form an electrochemical gradient ($\Delta\tilde{u}_{H^+}$) which creates the driving force for the flow of protons that is coupled to the ATP synthesis, catalyzed by the ATP synthetase (see scheme):

$$\Delta\tilde{u}_{H^+} = \Delta\Psi - Z\Delta pH$$



The ATP synthetase is a complex enzyme consisting of two main portions, F_1 an extrinsic membrane protein with catalytic activity and F_0 an integral membrane protein which has been postulated as the pathway by which protons traverse the membrane (Fillingame, 1980). The F_1 portion consists of five different subunits $\alpha, \beta, \gamma, \delta$ and ϵ , having a proposed stoichiometry of 3:3:1:1:1 (Gregory and Hess, 1981), and its catalytic and regulatory properties have been characterized to some extent (Caterrall et al. 1973; Kagawa et al. 1976; Knowles and Penefski, 1972; Takeshige et al. 1976). The F_0 portion consists of at least three subunits and it is not yet well established how it works (Schneider and Altendorf, 1982; Sone et al. 1978; Tzagoloff et al. 1968). In eukaryotic cells other subunits are present which may have a regulatory role on the activity of the whole complex during ATP synthesis, they are: The oligomycin sensitivity conferring protein (OSCP) (Mac Lennan and Tzagoloff, 1968), the DCCD binding protein (Nelson et al. 1977), the Factor B (Lam, et al. 1967; Sanadi, 1982), F_6 (Fessenden-Raden, 1972; Knowles et al., 1971), and the natural inhibitor protein (Pullman and Monroy, 1963). The ATP synthetase has been isolated from many sources: bacteria (Friedl et al. 1979; Sone et al. 1975), chloroplasts (Pick and Racker, 1979; Winget et al., 1977) and mitochondria (Ryrie, 1977; Serrano et al., 1976; Stiggall et al., 1978) and it is interesting to note that all of them are very similar in their structure suggesting its evolutionary importance.

Many experimental results have been explained by means of the chemiosmotic theory and attempts have been made in order to understand the molecular mechanism of ATP formation linked to electrochemical gradients: The direct model from Mitchell (1974) proposes that the protons flowing through the F_0 portion of the ATP synthetase create an extremely reactive phosphate which together with ADP in the active site forms ATP.

The indirect model of Boyer (1979) on the other hand proposes that the protons flowing through the ATP synthetase produce a conformational change (i.e. affinity changes for substrates and products) responsible of the release of the already synthesised ATP (the step which apparently requires more energy). In the first case the F_0 plays an active role during ATP synthesis but in the second that portion of the enzyme may not be required (Gresser et al., 1982; Sakamoto and Tonomura, 1983). It is important to provide direct experimental evidence which supports any of the proposed mechanisms. Since the inner mitochondrial membrane is composed of very complex multienzymatic systems, the best approach in this direction requires working with the purified ATP synthetase (or its isolated main portions or subunits) and studying its catalytic properties when re-incorporated in the membrane.

In this study I have developed a well controlled experimental model of the mitochondrial ATP synthetase. The first part is devoted to the purification and structural characterisation of the ATP synthetase from yeast mitochondria. The second deals with the functional reconstitution of the enzyme in a model membrane (i.e. liposomes) studying its two basic catalytic functions (i.e. ATP hydrolysis and ATP synthesis). Particular emphasis was placed on the coupling of these activities with electrochemical gradients. The ATP hydrolysis and the translocation of protons were measured using spectrofluorometric methods, and concomitantly a simple and very sensitive method to measure directly ATP synthesis was developed.

MATERIALS AND METHODS

1. Chemicals

ATP, ADP, AMP, NADH, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, cytochrome c, bovine serum albumin standard (Precimat), FCCP and oligomycin were from Boehringer (Mannheim, F.R.G.). Valinomycin and DTT were from Calbiochem (Frankfurt, F.R.G.). DCCD and 9-AA were from Fluka, A.G., (Neu-Ulm, F.R.G.). Asolectin was from ICN Pharmaceuticals, Inc. (Cleveland, U.S.A.). Anti-foam medium was from Roth GmbH (Karlsruhe, F.R.G.). α -Lysolecithin was from Sigma (München, F.R.G.). Nigericin was a gift from Eli Lilly & Co. (Indianapolis, U.S.A.). Oxonol VI was a gift from Dr. W.G. Hanstein, Bochum University (F.R.G.). ACMA was synthesized in our laboratory by B. Pevec. All other chemicals were analytical grade from E. Merck A.G. (Darmstadt, F.R.G.), J.T. Baker (Gross-Gerau, F.R.G.) or Serva GmbH (Heidelberg, F.R.G.).

2. Yeast culture conditions

The yeast wild-type strain YF (yeast foam ATCC 24967) was grown aerobically in a glucose-containing medium: 0.25% yeast extract, 1.2% $(\text{NH}_4)_2\text{SO}_4$, 1.0% KH_2PO_4 , 0.7% MgSO_4 , 0.5% NaCl , 0.4% CaCl_2 , 0.005% FeCl_3 , 1.0% Glucose. Four reaction tubes each with 2 ml medium, were incubated with the yeast (pre-culture I) and grown during 24 hours, with constant agitation. The temperature of the culture room was maintained constant at 28°C. For the pre-culture II, four Erlenmeyer flasks (5 liter) each containing 500 ml of the glucose-containing medium and 2 ml of pre-culture I, were incubated during 24 hours, with vigorous agitation at the same room temperature (28°C). The pre-culture II was added to a 150 liter capacity fermenter (Fermenter model F150, New Brunswick Scientific Co., New Jersey, U.S.A.) filled with about 100 liter culture medium

without glucose, but having 2% ethanol as a carbon source. The culture was agitated constantly at 2,000 rpm and foaming prevented by the addition of anti-foam medium. The air flow pressure was maintained at 40 liter/min and the temperature at 30°C. After 24 hours the cells were harvested with a Westphalia Separator (Oelde, Westfalen, F.R.G.) connected directly to the fermenter. The resulting cell suspension (about 6 liter) was washed twice with water. The yeast pellet was kept cold for not longer than two days. Usually 1.2 kg of yeast were obtained (wet weight).

3. Preparation of the mitochondria

For the large scale preparation of mitochondria, the method of Guérin et al. (1977) was adapted as follows: A Dyno Mill model KDL (Basel, Switzerland) equipped with a 600 ml continuous flow breakage glass chamber, four 6.4 cm polymethane rotating agitator disks, and a 0.05 mm dynamic slit separator. The chamber was filled with 520 ml of glass beads (0.5 mm), and cooled by circulating an ethylene glycol/water mixture (1:1, v/v) from a 80 liter capacity cold bath (flow rate 300 liter/hour).

The yeast cells were suspended in the following solution: 0.65 M Sorbitol, 0.1 mM EDTA, 1% Albumin, adjusted to pH 6.3 with KOH (S-E-A medium), to yield a 60% suspension (wet weight). This suspension was kept ice-cold during the whole preparation. The cells were driven through the pre-cooled mill by a peristaltic pump (60-80 ml/min) and when the breakage chamber was full, the driving motor was switched on (stirring speed 3, 000 rpm), while pumping continued at the same flow rate. Broken cells were caught in a 5 liter glass beaker kept on ice. The temperature of the suspension at the exit point was about 15°C. After the yeast suspension had passed through the mill, the chamber was washed with 1 liter

cold S-E-A medium obtaining in this way almost all the broken cells out of the chamber. Immediately 1.5 liter more of the S-E-A medium were added and the pH adjusted to 6.3 with KOH (30%). Estimation of cell breakage was made by means of phase microscopy (> 90%).

The broken cells were centrifuged at 2,750 x g for 30 min. Only the brownish supernatant was sucked off with a syringe, and centrifuged again at 4,500 x g for 30 min. The supernatant was carefully decanted and centrifuged at 10,900 x g for 30 min to collect the mitochondria. The supernatant was discarded, the fat deposits were wiped off and the mitochondria resuspended in a glass homogenizer with Teflon pestle up to 1 liter in S-E-A medium. This homogenate was centrifuged at 27,000 x g for 30 min. The resulting mitochondria were resuspended in the same way as above in a final volume of 500 ml and immediately frozen in portions of 100 ml (1.5-2 g protein) and stored at -70°C for several weeks.

4. Submitochondrial particles preparation

Submitochondrial particles were prepared as described by Harmon (1982) with the following modifications: 200 ml of frozen mitochondria were thawed and diluted with 0.25 M Sorbitol, 10 mM Tris-H₂SO₄ pH 7.5 (S-T buffer), to a final volume of 480 ml (about 10 mg protein/ml). The diluted mitochondria were centrifuged at 27,000 x g for 15 min and the resulting pellet resuspended in 120 ml of S-T buffer with a glass homogenizator and Teflon pestle. The mitochondria were then sonicated for 30 sec in a 40 ml stainless steel vessel at 200 Watt in a Branson Sonifier (Model B-30, Danbury, Conn. U.S.A.), with a 1/2 inch diameter tip. The vessel was maintained in ice with NaCl to prevent overheating. To the sonicated mixture 120 ml of the S-T buffer were added and centrifuged at 22,000 x g for 10 min. The supernatant was carefully decanted taking care not to remove any portion of

the dense pellet material. The supernatant was centrifuged at 80,000 x g for 30 min. The pellets were resuspended in S-T buffer with 150 mM KCl and centrifuged as above. The pellets were resuspended in S-T buffer and centrifuged at 22,000 x g for 15 min. The supernatant was carefully decanted and centrifuged at 105,000 x g for 30 min. The sub-mitochondrial particles were resuspended in S-T buffer and stored on ice for about 1 hour, before the solubilisation of the ATP synthetase.

5. ATP synthetase preparation

The ATP synthetase was prepared by the α -Lysolecithin extraction method of Penin et al. (1982). The following modifications were introduced: The submitochondrial particles were diluted to a protein concentration of 10 mg/ml with the S-T buffer containing 10 mM MgSO_4 and 2.6 mM CaCl_2 . α -Lysolecithin was added to give a final concentration of 0.2%. After 30 min incubation at 0°C with magnetic stirring, an equal volume of 100 mM MES-KOH buffer (pH 6.5) was added and incubated 20 min at 0°C. The suspension was centrifuged at 105,000 x g for 15 min. The supernatant was carefully decanted and centrifuged at 155,000 x g for 90 min. The pellets containing the ATP synthetase were homogenised with a glass homogeniser in a minimum volume of S-T buffer. Aliquots of these pellets were stored at -70°C. All operations starting from the breakage of the yeast cells were performed at 4°C.

6. Preparation of a crude F_0

The crude F_0 preparation was done according to Tzagoloff (1971) as follows: submitochondrial particles (20 mg/ml) were resuspended in a buffer containing 0.25 M Sorbitol, 0.1 mM DTT, 10 mM Tris-HCl (pH 7.5). An equal volume of 6 M NaBr was added and the pH immediately adjusted to pH 7.5 with Tris. The sus-

pension was incubated at 0°C for 20 min and centrifuged at 164,000 x g for 20 min. The resulting floating layer was carefully removed with a spatula and washed under exactly the same conditions as described above. The twice extracted membranes were washed with a buffer containing 0.25 M Sorbitol, 10 mM Tris-HCl (pH 7.5) and the final precipitate (crude F_O) was suspended in the same buffer at a protein concentration of 3 mg/ml.

7. Preparation of liposomes

Soy-bean phospholipids (Asolectin) were purified by the method of Kagawa and Racker (1971) and stored in CHCl₃ at -30°C until use. In a typical preparation, 100 mg of the asolectin suspension with the same amount of CHCl₃ were dried in a round-bottom flask on a rotatory evaporator. Two ml of buffer (1 mM EDTA; 150 mM KCl; 50 mM Tricine pH 8.2) were added and evacuated under a stream of N₂. The flask was sealed and left overnight at 4°C. The lipids film was removed from the flask walls with the help of two glass beads; the flask was washed with another two ml of the same buffer. The mixture was sonicated under a N₂ stream using a Branson sonifier B-30 with 1/2 inch tip. The sonication time was 23 min with intervals every 48 sec for 12 sec and the mixture was maintained in an ice-water bath. Because of the deterioration of the tip, the liposomes were filtered through a Bio Rad Unipore (0.22/μm) membrane filter in order to eliminate possible tip debris and stored at 4°C until use. The liposomes were always prepared immediately before the experiments.

In the cases in which bacteriorhodopsin was used, it was added to the lipid mixture before sonication and the procedure was the same as above. The lipid: protein ratio was 50:1.

Bacteriorhodopsin was purified according to Oesterhelt and Stoebenius, 1974.

7.1 Reconstitution of the ATP synthetase

The ATP synthetase was incorporated in the liposomes using the method of Kasahara and Hinkel (1977). To the liposomes was added the desired amount of ATP synthetase protein (typically 1 mg/2 ml liposomes) mixed on a Vortex mixer and frozen in acetone-dry ice. After being thawed at room temperature the liposomes were sonicated in a bath-type sonicator (Branson Model B-220) for 30 sec or until clarity was obtained. The suspension was again frozen, thawed and sonicated. The reconstituted liposomes were used immediately for experiments. For some experiments the extravesicular concentration of K^+ was reduced virtually to zero by applying the liposomes onto a PM-10 column (Pharmacia, Uppsala, Sweden) pre-equilibrated with 1 mM EDTA, 50 mM Tricine pH 8.2.

7.2 Reconstitution of the F_o

The liposomes were prepared as described by Sone et al. (1978, 1981) as follows: to 80 mg partially purified asolectin 2 ml of a buffer containing 7.5 mM DTT, 0.2 mM EDTA, 1.6% cholate, 0.8% deoxycholate, 15 mM Tricine-NaOH (pH 8.0) were added. After suspension with a Vortex mixer, the mixture was sonicated during 10 min with intervals of 0.5 sec at 200 Watt. To 1 ml of the resulting suspension 0.5 mg of the crude F_o were added and dialysed against 1000 volume of a buffer containing 2.5 mM $MgSO_4$, 0.25 mM DTT, 0.2 mM EDTA, 10 mM Tricine-NaOH (pH 8.0), during 18 hr changing the buffer after 4 and 14 hrs. To the resulting liposomes the same amount of a 20 mM phosphate buffer (pH 7.0) containing 0.4 M KCl was added in order to load the liposomes with K^+ . After 10 sec sonication as described above, the mixture was frozen at $-80^\circ C$, thawed at room temperature for 20 min and sonicated again as before. For the proton uptake measurement usually 50 μ l of the K^+ -loaded F_o liposomes were added to a cuvette

containing 1950 μ l of 0.2 M Na_2SO_4 , 5 mM MgSO_4 , 0.5 mM Tricine-NaOH (pH 8.0) and the pH change was followed with a combined micro glass electrode (Metrohm, AG, Herisau, Switzerland).

8. SDS Gel electrophoresis

The gel electrophoresis was performed by the procedure of Laemmli (1970) using a polyacrylamide gel gradient. Separation gradient gels were formed in slabs from equal volumes of 10% acrylamide (9.98% acrylamide, 0.27% bisacrylamide, 0.04% APS, 0.05% TEMED, 0.1% SDS, 2 mM EDTA, 375 mM Tris-HCl pH 8.8) and 17.5% acrylamide (17.4% acrylamide, 0.046% bisacrylamide, 0.04% APS, 0.05% TEMED, 0.1% SDS, 2 mM EDTA, 375 mM Tris-HCl pH 8.8). Polymerization was carried out at room temperature. 4.5% acrylamide stacking gels were overlaid (4.51% acrylamide, 0.12% bisacrylamide, 0.09% APS, 0.06% TEMED, 0.1% SDS, 2 mM EDTA, 125 mM Tris-HCl pH 6.8). The gels were usually stored overnight at 4°C. The samples were solubilized for 15 min at room temperature in: 2% SDS, 2 mM EDTA, 1% mercaptoethanol, 10% glycerol (v/v), 1% bromophenol blue, 50 mM Tris-HCl pH 6.8. Electrophoresis was carried out at room temperature at 40 mA for 4 hours. The running buffer contained 0.1% SDS, 384 mM glycerol, 2 mM EDTA, 50 mM Tris. The gels were fixed and stained in 45.4% methanol, 9.2% acetic acid and 0.25% Serva blue R-250 (Serva Heidelberg, F.R.G.) during 30 min and destained in 5.0% methanol, 7.5% acetic acid.

As molecular weight standards the following proteins were used: Phosphorylase b (94,000) Albumin (67,000), Ovalbumin (43,000), Carbonic anhydrase (30,000), Trypsin inhibitor (20,100), and α -Lactalbumin (14,400), from Pharmacia (Uppsala, Sweden).

9. Protein determination

The protein concentration was determined by the method of Lowry et al. (1951) using Precimat as standard.

10. Measurement of oxidative phosphorylation in mitochondria

Oxidative phosphorylation was measured in freshly prepared mitochondria as described by Somlo et al. (1977), by means of a Clark electrode in a thermostable chamber at 30°C.

10 μ l of ethanol were added as substrate to 1.5 ml of the assay buffer (0.25 M Sorbitol, 1 mM EDTA, 10 mM KH_2PO_4 , 0.1% albumin, adjusted to pH 6.3 with KOH). About 1-2 mg of mitochondrial protein were injected in the chamber giving a final volume of 1.7 ml. After about 3 min 10 μ l of a 17 mM ADP solution were added and the consumption of oxygen measured. The respiration rate and respiratory control were calculated by the method of Chance and Williams (1955).

11. Enzymatic assays

11.1. ATPase

ATP hydrolysis was measured photometrically at 334 nm using an ATP-regenerating system coupled to the oxidation of NADH (Pullman et al. 1960) in the following reaction mixture (1 ml): 2 mM PEP, 0.4 mM NADH, 50 μ g PK, 25 μ g LDH, 3 mM MgCl_2 , 1 mM KCN, 2 mM ATP and 50 mM Tris-acetate adjusted to pH 8.0. Usually 10 μ l of the diluted protein were added. The extinction coefficient used was 6.18 $\mu\text{mol}^{-1}\text{cm}^2$.

For the steady-state kinetics, the reaction mixture was modified as follows: 4 mM PEP, 0.5 mM NADH, 50 μ g PK, 50 μ g LDH, 1 mM MgCl_2 , and 0.2 M HEPES adjusted to pH 7.0. The ATP concentration was varied from 0.005 to 2.5 mM.

11.2. NADH cytochrome c reductase

The activity of the NADH cytochrome c reductase was measured according to Hatefi and Rieske (1967) as follows: To 1 ml of reaction mixture containing 2 mM NaN_3 , 5 μM cytochrome c, 10 μM EDTA, 250 μM NADH and 20 mM KH_2PO_4 adjusted to pH 8.0, 10-20 μl of diluted protein were added. The absorbance change at 546 nm was recorded and the extinction coefficient used was 18.5 $\mu\text{mol}^{-1}.\text{cm}^2$.

11.3. Cytochrome c oxidase

The activity of the cytochrome c oxidase was measured as follows: 50 μl of reduced cytochrome c (about 50 mg/ml) were added to 850 μl of a buffer containing 1 mM EDTA, 3 mM KCl and 10 mM KH_2PO_4 adjusted to pH 7.0. The reaction was started with 10 μl of the diluted protein sample. The absorbance change was monitored at 546 nm, and the extinction coefficient used was 18.5 $\mu\text{mol}^{-1}.\text{cm}^2$. In order to make the substrates more accessible to the enzyme, 10 μl of a 1% Triton X-100 solution were added to those samples containing sub-mitochondrial particles.

11.4. Adenylate kinase

The activity of the adenylate kinase was measured according to Bergmeyer (1974) modified as follows: 10 μl (about 10 μg protein) of the protein sample were added to a reaction mixture containing 5 mM MgCl_2 , 5 mM PEP, 0.3 mM NADH, 11 I.U. LDH, 4 I.U. PK, 2.5 mM AMP, 2.5 mM ATP, 100 mM Tris-acetate adjusted to pH 8.0. The activity was monitored at 334 nm using an extinction coefficient of 6.18 $\mu\text{mol}^{-1}.\text{cm}^2$.

All enzymatic assays were performed at 25°C in an Eppendorf photometer Model 1101 M (Netheler & Hinz GmbH, Hamburg, F.R.G.).

12. Proton translocation induced by ATP hydrolysis.

ATP-dependent proton translocation was measured fluorometrically monitoring the quenching of the fluorescent acridine dye ACMA as reported by Schneider and Altendorf (1982) with the following modification: The reconstituted liposomes were formed as described above but without KCl. The assay medium contained in a final volume of 2 ml 10 mM MgCl_2 , 300 mM KCl, 2 mM Tricine-NaOH pH 8.0, $1.6\text{ }\mu\text{M}$ Valinomycin, $2\text{ }\mu\text{M}$ ACMA, 2 mM ATP and $25\text{ }\mu\text{g}$ ATP synthetase protein. The quenching of the fluorescence was monitored by means of a home-made fluorometer using a Xenon-Hg lamp as light source and the wavelengths of 405 nm for excitation and 494 nm for emission.

13. ATP-synthesis in liposomes containing the ATP synthetase

13.1. ATP synthesis driven by a proton gradient induced with bacteriorhodopsin.

The formation of ATP was measured in the following reaction mixture (1 ml): 5 mM MgSO_4 , 5 mM KH_2PO_4 , 50 mM Tricine-NaOH pH 7.5 and liposomes containing $180\text{ }\mu\text{g}$ of bacteriorhodopsin and $150\text{ }\mu\text{g}$ of ATP synthetase. The reaction vial was illuminated with a Leitz CA 2500 projector during 1 minute in the presence of $2\text{ }\mu\text{M}$ of Valinomycin; after this period of time ADP was added to a final concentration of 1 mM and aliquots of $240\text{ }\mu\text{l}$ were taken at specified times; The reaction was stopped by addition of $100\text{ }\mu\text{l}$ 35% HClO_4 . After centrifugation at $12,000 \times g$ an aliquot of the supernatant was neutralised with NaOH and the ATP content determined using reverse-phase HPLC.

13.2. ATP synthesis driven by a valinomycin induced diffusion potential

The formation of ATP was measured under the same conditions as described above except that the external concentration of KCl was varied and the ionic strength was kept constant with the respective concentration of LiCl. After 3 min incubation with 2 μ M of Valinomycin ADP was added to a final concentration of 1 mM. The rest of the procedure was as above. The assays were done at room temperature and in all conditions blank liposomes (without ATP synthetase) were assayed as control.

14. Determination of ATP

Samples were analysed using an isocratic elution mode of reverse-phase HPLC. The eluant was 50 mM sodium phosphate buffer (pH 5.0) and the flow rate 1 ml/min. Chromatography was carried out at constant pressure with a Beckman (Beckman Instruments, Inc. Berkeley, U.S.A.) Model 112 solvent delivery system, equipped with a Beckman Model 160 absorbance detector (detection wave length 254 nm) and a Shimadzu (Kyoto, Japan) Chromatopac C-R2AX data processor. Sample injections were made with a Waters Assoc. (Milford, U.S.A.) WISP-710B automatic sample injection system. A prepacked Waters μ Bondapack C₁₈ (3.9 mm I.D. x 30 cm, 10 μ m particle size) column was used. Samples were maintained at 4°C, and the determinations were done at room temperature.

15. Electron microscopy

Samples for electron microscopy were submitochondrial particles and liposomes reconstituted with bacteriorhodopsin and/or ATP synthetase. The freeze-etching preparation was done according to Müller et al. (1980) using the quick-freeze

method. The frozen samples were broken and replicated (Pt/C) in a Balzer BAF 300 (Balzers AG, Wetzlar, F.R.G.) freeze etching device. The clean replicas were observed and photographed in a Siemens Model 101 electron microscope at 80 KV. The negative staining procedure was done using 2% phosphotungstate at pH 6.8 in copper grids (400 mesh) coated with carbon. The samples were observed in the same microscope as above at 80 KV.

RESULTS

1. Purification of the ATP synthetase from yeast mitochondria

1.1. Characterisation of the yeast mitochondria

It is well known that yeast cells grown aerobically under conditions of glucose repression have poorly developed mitochondria, with low respiratory activities (Tzagoloff, 1971). It was found that when mitochondria were prepared from yeast grown in these conditions, no activation of the respiratory rate induced by ADP was seen. In order to obtain a better respiratory control, the yeast cells were grown aerobically in the presence of ethanol as carbon source, and indeed under these conditions the yeast cells developed large amount of mitochondria (Fig. 1).

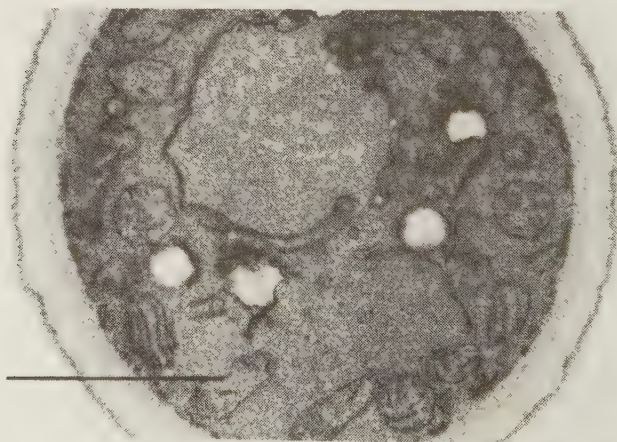


Fig. 1. Electron micrograph of a yeast cell.
Bar represents 1, μ m.

As can be clearly seen in Fig. 2, the respiratory rate of mitochondria prepared from these cells can be stimulated by ADP and this effect is completely blocked by oligomycin, an ATP synthetase inhibitor. Using ethanol as substrate an ADP:O ratio of 1.7 and a respiratory control of 3.9 were obtained. A very similar result was obtained using succinate as substrate. This mitochondrial preparation was used as starting material in order to purify the ATP synthetase complex.

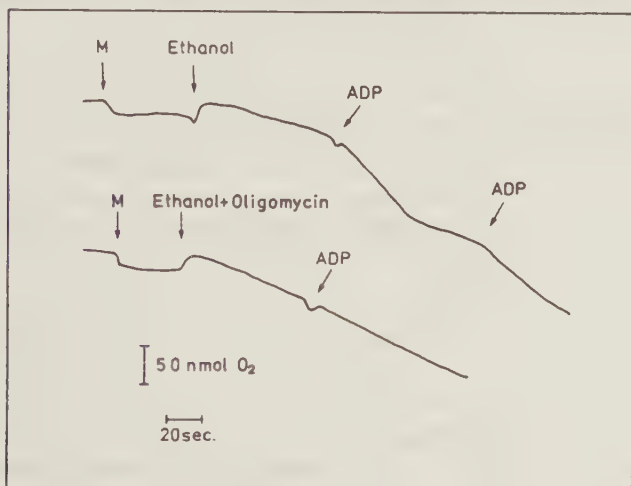


Fig. 2. Effect of oligomycin on the respiratory control of yeast mitochondria.

The experimental conditions for the measurement of the oxidative phosphorylation are described in Materials and Methods. Oligomycin was added from an ethanolic solution given a final concentration of 10 μ g/mg of mitochondrial protein. M = mitochondria.

1.2. Solubilization and polypeptide composition of the ATP synthetase.

Several detergents were tested in order to solubilize a functional ATP synthetase: Triton X-100 (Ryrie, 1977), a mixture of sodium cholate and octylglucoside (Rott and Nelson, 1981), and the Zwittergent TM-314 (Malpartida and Serrano, 1980). As can be seen in Table 1 all these preparations yield very low specific activities of ATP hydrolysis.

Table 1: Effect of some detergents on the solubilization of the ATP synthetase.

	Protein total (mg)	ATPase activity /umol/min/mg prot.
Triton X-100 (1)	11.3	7.8
Na-cholate-octyl- glucoside (2)	15.0	3.1
Zwittergent TM-314 (3)	3.3	10.5

The solubilizations were done according to (1) Ryrie (1977), (2) Rott and Nelson (1981), (3) Malpartida and Serrano (1980).

The best specific activity (mean value of 15 purifications was 16.5 ± 1.2 /umol/min/mg protein) and sensitivity to inhibitors was obtained with α -lysolecithin (Hughes et al. 1982; Penin et al. 1982) and a representative purification using this detergent is summarised in Table 2. The inhibitory effect of oligomycin (10 /ug/ml) on the ATPase activity was very high in the α -lysolecithin preparation (> 90%) and a similarly high inhibition was obtained using DCCD (100 /uM). Oligomycin and DCCD interfere with the passage of protons through the F_o , thus preventing ATP hydrolysis and synthesis, and therefore oligomycin and DCCD sensitivity are related to a correct

assembly of the ATP synthetase. In Table 2 it is also shown that other enzymatic activities are detected but they are relatively low and even decrease along the purification steps, indicating a relatively specific solubilisation of the ATP synthetase by α -lysolecithin. Many attempts were made in order to completely remove these contaminants (gradient centrifugation, and gel filtration chromatography) but none of them was successful, without affecting the ATP hydrolytic activity and the sensitivity to oligomycin and DCCD of the ATP synthetase.

Table 2: Purification of the ATP synthetase from yeast mitochondria.

	Mitochondria	Submitochondrial particles	ATP synthetase
Total protein (mg)	2,000	392	5
ATPase activity	2.4	4.0	17.1
Purification fold	1.0	1.6	7.1
NADH-cytochrome c reductase	n.d.	0.51	0.17
Cytochrome c oxidase	n.d.	4.59	0.18
Adenylate kinase	n.d.	1.81	0.21

Experimental details of the purification and the enzymatic assays are described in Materials and Methods. n.d. = not determined. All enzymatic activities are expressed in $\mu\text{mol/min/mg protein}$.

The polypeptide composition and homogeneity of the ATP synthetase preparation was determined by SDS-polyacrylamide gel electrophoresis. In Fig. 3 the five subunits of the F_1 portion of the ATP synthetase complex are easily identified using purified F_1 (Takeshige et al., 1976) as standard. Some of the remaining bands are related to other subunits present

in the ATP synthetase from mitochondria: OSCP (23,000); F_B (14,600); Factor 6 (12,500); natural inhibitor protein (7,000) and DCCD binding protein (8,000). Some of these polypeptides are presumably components of the F_O portion. There are some reports which assign at least 18 different polypeptides to the ATP synthetase complex (Ludwig et al., 1980). To identify the subunits of the F_O portion from eukaryotic organisms unequivocally it will be necessary to purify and reconstitute this portion of the enzyme independently of the rest.

The crude extraction of F_O after SDS polyacrylamide gel electrophoresis did not show the main bands of the F_1 and exhibited no detectable ATPase activity (not shown). However, some of the identified polypeptides can be attributed to the F_O portion of the ATP synthetase (Alfonzo et al., 1981). This will be confirmed later when the proton conducting properties of this preparation are examined.

A better starting material for the purification of the F_O , using the same extraction procedure, should be the purified ATP synthetase instead of the submitochondrial particles (Glaser et al., 1980).

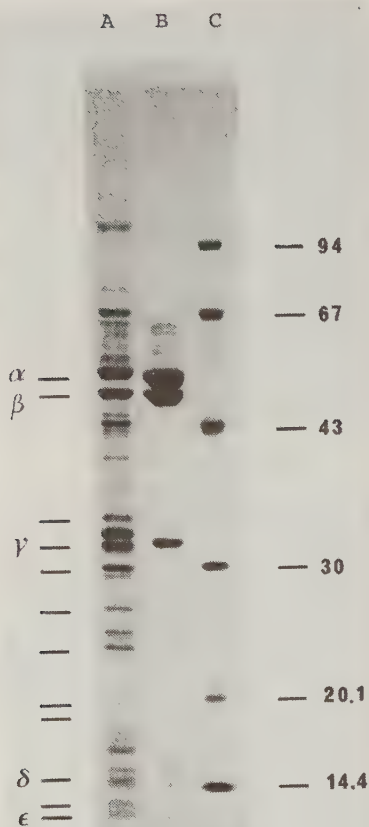


Fig. 3: SDS-polyacrylamide gel electrophoresis.

(A) ATP synthetase; (B) F₁; (C) M.W. standards.

2. Electron microscopy of the reconstituted ATP synthetase in liposomes.

A positive identification of membrane proteins reconstituted in liposomes can be accomplished by means of electron microscopy. Two techniques were used for this purpose: negative staining and freeze-etching. Fig. 4a shows a negative staining of asolectin liposomes and Fig. 4b shows the same kind of liposomes reconstituted with the ATP synthetase preparation by a freeze-thaw-sonication procedure. By comparing these pictures it can be seen that the reconstituted liposomes have regular structures on their surfaces which are not observed in blank liposomes. These structures or protein particles can be better identified by freeze-etching in Fig. 5b. The asolectin liposomes in Fig. 5a do not have those characteristic particles. Interestingly, Fig. 5c shows that protein particles of the same size (about 90 Å) are also clearly observed in native submitochondrial particles and presumably they represent the F_1 part of the ATP synthetase (Fernández-Morán et al., 1964; Kagawa and Racker, 1966).

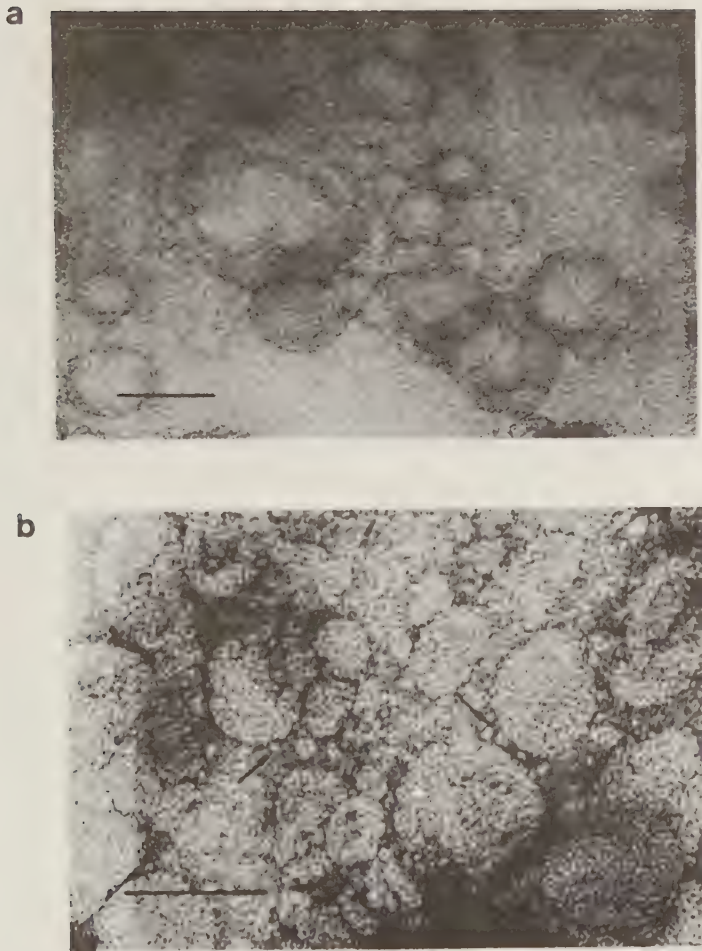


Fig. 4: Negative staining of reconstituted ATP synthetase in asolectin liposomes.

a) asolectin liposomes, b) ATP synthetase liposomes. The details for the preparation of the liposomes, reconstitution and negative staining are described in Materials and Methods. Bars are equivalent to 100 nm.

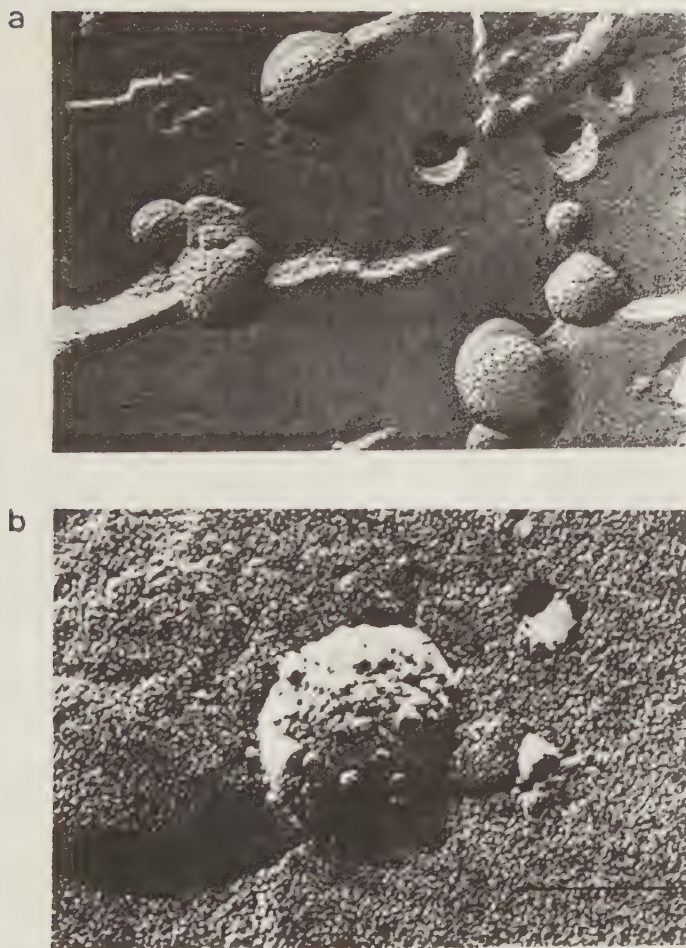


Fig. 5: Freeze-etching electron micrographs of the reconstituted ATP synthetase in liposomes.

a) Asolectin liposomes, b) ATP synthetase liposomes, c) submitochondrial particles.

Bars are equivalent to 100 nm.

C



3. ATP hydrolysis.

3.1. Steady-state kinetics of the solubilized and reconstituted ATP synthetase.

The ATP hydrolysis catalysed by submitochondrial particles, the α -lysolecithin solubilized ATP synthetase, the enzyme re-incorporated in liposomes and the free F_1 was compared under steady-state conditions. The kinetic constants in Table 3 show that the free F_1 has a $V_{\max}$ which is more than one order of magnitude faster than the membrane bound forms of the enzyme. This difference apart from representing exclusively the purity of the different preparations, may indicate an activation of the free F_1 as reported by others (Penefsky et al., 1960). On the other hand the affinity of the free F_1 for Mg-ATP was found to be slightly lower as indicated by its higher K_m . However, the submitochondrial particles containing the native enzyme gave a very similar K_m in comparison to the solubilized or reconstituted ATP synthetase.

The Hill plot in Fig. 6 shows that the reported negative cooperativity for ATP hydrolysis (Gresser et al., 1982; Wieker

Table 3: Kinetic constants for ATP hydrolysis.

	n_H	$V_{\max}$ ($\mu\text{mol/min/mg prot}$)	K_m μM
F_1	0.71	119.60	170.4
ATP synthetase	0.63	10.54	61.7
ATP synthetase liposomes	0.73	6.61	63.6
SMP	0.77	3.12	105.4

Details of the experimental conditions are under Materials and Methods.

and Hess, in press) is conserved in all the different forms of the enzyme as indicated by a n_H value of approximately 0.7.

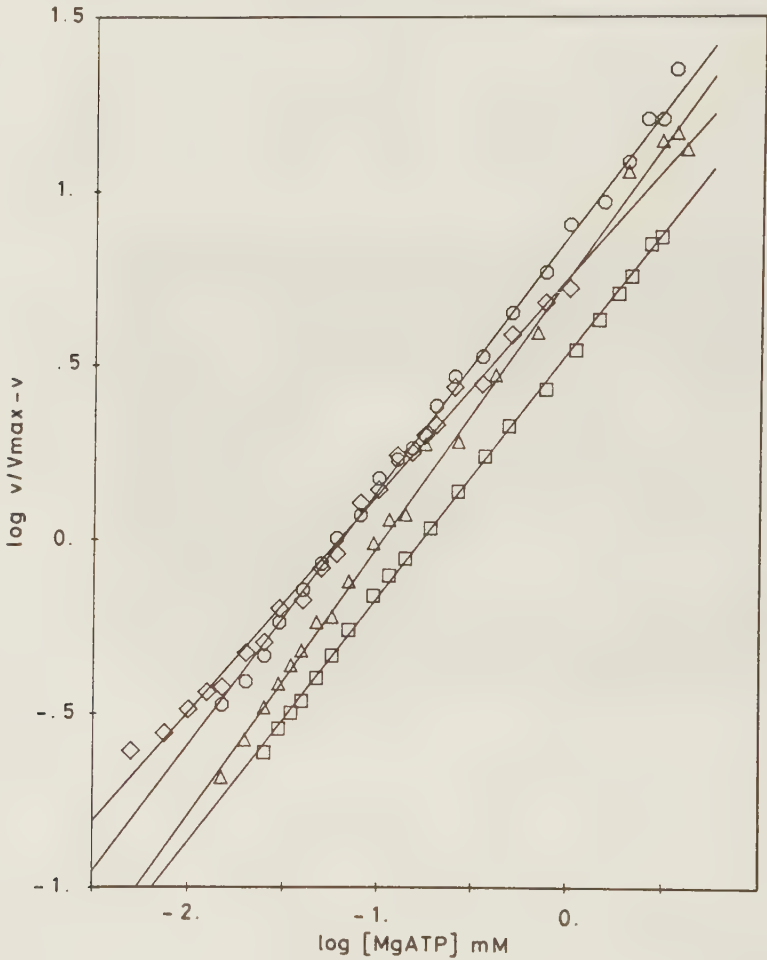


Fig. 6: Hill plot for ATP hydrolysis using different forms of the mitochondrial ATPase.

◇ ATP synthetase

□ F₁

△ SMP

○ ATP synthetase-liposomes

3.2. Proton Translocation coupled to ATP hydrolysis.

In order to test whether the reconstituted ATP synthetase was able to pump protons when it hydrolyses ATP, the ATP-dependent proton translocating activity was monitored by means of the fluorescent dye ACMA. This hydrophobic probe is dissolved in the lipidic phase of the membrane and depending upon the proton concentration in the bulk solution inside the liposomes, the 9-amino group of the fluorophore can be protonated resulting in a quenching of its fluorescence (Kraayenhof, 1980; Schuldiner et al., 1972). As shown in Fig. 7 trace (A), the addition of ATP to the ATP synthetase liposomes produces a quenching of the ACMA fluorescence of approximately 35%. Once the signal reached equilibrium the addition of nigericin (5 μ g/ml), a K^+H^+ exchanger, reversed the fluorescence change indicating that protons have indeed been taken up into the liposomes. Accordingly the quenching of the fluorescence was abolished by prior addition of the inhibitors oligomycin (10 μ g/ml) and DCCD (100 μ M) or the ionophore nigericin (5 μ g/ml) (Fig. 7 traces B,C and D respectively). The fact that the fluorescence does not return to the original level in (A) is due to a dilution and pH effect of the ATP addition as can be seen in trace (E), a control experiment with blank liposomes.

The experiments described above were done in the presence of the K^+ -selective ionophore valinomycin. This is advantageous since by increasing the permeability to K^+ the liposomes were hyperpolarized (i.e. inside more negative) creating a driving force that facilitates the take-up of protons.

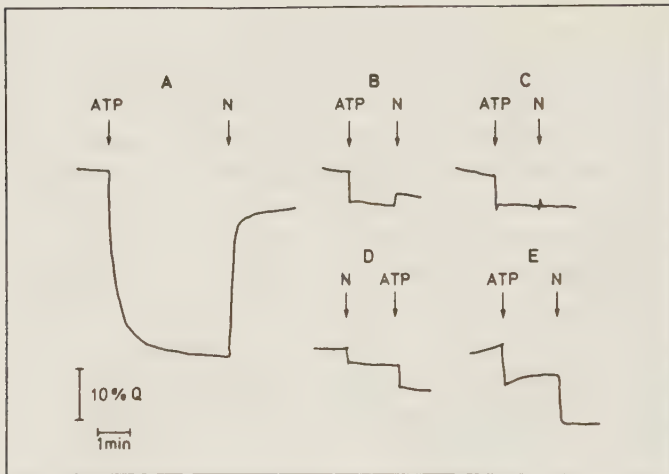


Fig. 7: ATP-induced quenching of the ACMA fluorescence in liposomes reconstituted with the ATP synthetase. The experimental details are described in Materials and Methods. The protein concentration was 25 $\mu\text{g/ml}$ and the final concentration of ATP was 2 mM. N = nigericin.

4. ATP Synthesis

4.1. Light-driven proton pumping in liposomes reconstituted with bacteriorhodopsin.

Bacteriorhodopsin, a light-dependent proton pump (Oesterhelt and Stoeckenius, 1974), can be used as a tool when reconstituted in liposomes. Following illumination bacteriorhodopsin can generate an electrochemical proton gradient, large enough to be used by another energy-transducing protein (i.e. ATP synthetase) in order to synthesise ATP (Racker and Stoeckenius, 1974).

Fig. 8 shows the fluorescence of 9-AA in liposomes reconstituted with bacteriorhodopsin. Under illumination the proton pumping activity of bacteriorhodopsin is activated and an accumulation of protons in the interior of the liposomes is observed as a quenching of the fluorescence of 9-AA (by the same mechanism described for ACMA in Chapter 3). When the light is turned off, the bacteriorhodopsin stops pumping protons, and due to the proton leakage through the membrane of the liposomes the fluorescence is recovered. This result confirms that bacteriorhodopsin is able to generate a proton gradient which might be used by the ATP synthetase to form ATP. It is interesting to note that DCCD inhibits the proton pumping activity of bacteriorhodopsin at the same concentration used to inhibit the proton-translocating activity of the ATP synthetase. This finding indicates that easily accessible carboxyl groups may be involved in the mechanism of proton pumping as proposed for other proton-translocating membrane proteins (Lenaz et al., 1982; Okamoto et al., 1977; Sebald, 1980).

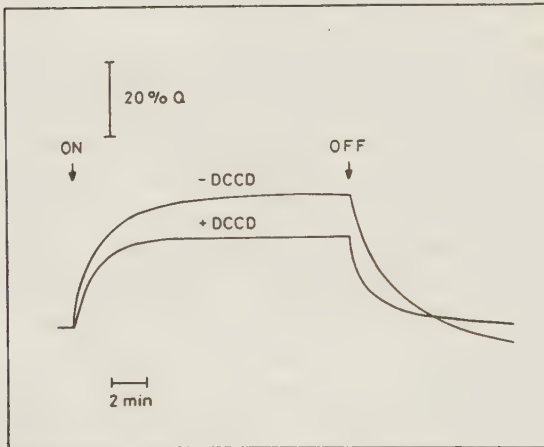


Fig. 8: Light dependent proton uptake in liposomes reconstituted with bacteriorhodopsin. The liposomes were prepared as described in Materials and Methods. The assay buffer contained: 0.1 mg/ml bacteriorhodopsin, 2 mM MgSO_4 , 2 μM 9-AA and 25 mM glycylglycine pH 8.2. Before turning the light on (filtered through a 570 nm filter), the liposomes were preincubated 5 min with 0.3 μM valinomycin. The excitation light from a second lamp was passed through an Eppendorf filter (313-366 nm) and the fluorescence passing another Eppendorf filter (430-470 nm) was monitored. The liposomes in the presence of DCCD were pre-incubated with this inhibitor for 20 min before the addition of valinomycin.

4.2. ATP determination by means of reverse phase HPLC.

A method was developed to measure directly the net amount of ATP synthesised using reverse phase HPLC. In the chromatography profile shown in Fig. 9 it can be clearly seen that ATP and ADP are well resolved, and therefore it is possible to measure accurately the amount of ATP present in samples obtained from experiments of ATP synthesis.

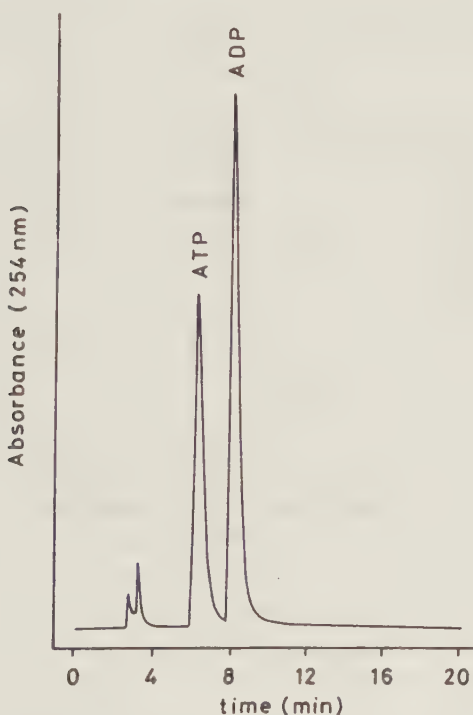


Fig. 9: Reverse phase HPLC of ATP and ADP.

The separation of a mixture of ATP (12.5 nmol) and ADP (15 nmol) was done according to the procedure described in Materials and Methods.

In order to be able to quantify the ATP formed in these sets of experiments, known amounts of ATP were injected in the HPLC system and a calibration curve obtained. Fig. 10 shows a calibration curve for ATP in the range from 50 to 250 pmoles and it indicates the high accuracy and sensitivity of the determinations.

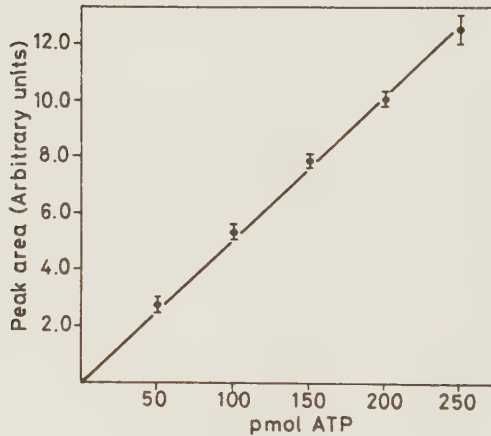


Fig. 10: Calibration curve for ATP using reverse phase HPLC. The ATP standards were prepared in the same conditions of the samples obtained from the ATP synthesis experiments and measured as described in Materials and Methods. The vertical bars represent the S.D. from 10 independent determinations.

4.3. Synthesis of ATP in liposomes reconstituted with the ATP synthetase and bacteriorhodopsin.

As shown in paragraph 4.1, the reconstituted liposomes with bacteriorhodopsin were able to generate a proton gradient upon illumination. Therefore the electrochemical gradient generated could support the synthesis of ATP from ADP and P_i (Racker and Stoeckenius, 1974). In order to verify this finding, liposomes containing bacteriorhodopsin were co-reconstituted with the ATP synthetase and the net synthesis of ATP was measured under constant illumination.

Fig. 11 shows that ATP is indeed synthesised upon illumination of the liposomes in the presence of ADP and P_i . It can also be seen that over a period of 10 min, the synthesis of ATP by the ATP synthetase depends linearly on the time of illumination giving a rate of 19 nmol ATP/min/mg of ATP synthetase protein. In parallel a control experiment was done in the absence of the ATP synthetase. In this case a very small amount of ATP was detected (0.26 nmol), but this did not change as a function of the illumination time and it is therefore attributable to a small ATP contamination of the ADP obtained from commercial sources. Accordingly, at each time of illumination of the liposomes in the presence of the ATP synthetase and bacteriorhodopsin, the background in the absence of the enzyme was subtracted. The amount of ATP detected at time zero was practically identical with that obtained in the liposomes reconstituted only with bacteriorhodopsin.

In order to further stress the specificity of the observed synthesis of ATP, measurements were done in parallel to the control experiment in the presence of the inhibitors oligomycin and DCCD. Both inhibitors reduced the ATP synthesis (Table 4). By abolishing the proton gradient, the H^+ -ionophore FCCP also inhibited ATP synthesis. However, it is

interesting to notice that with all the inhibitors used (including the dark control) the inhibition decreases as a function of time. This observation will be discussed later in more detail.

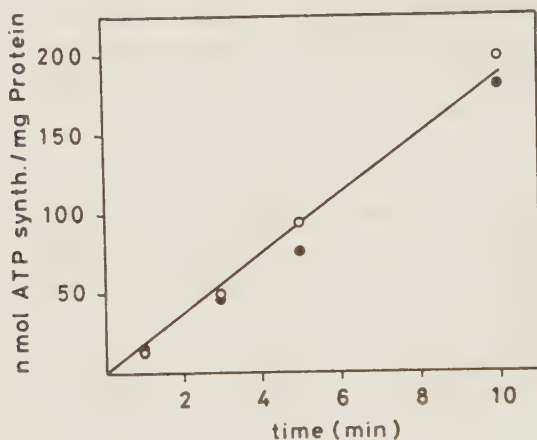


Fig. 11: Synthesis of ATP during illumination of liposomes containing bacteriorhodopsin and the ATP synthetase. The reconstitution and experimental details are described under Materials and Methods. The reconstituted liposomes contained 185 and 163 μ g of bacteriorhodopsin and ATP synthetase respectively. The two different symbols in the figure represent two independent determinations using two different preparations of the ATP synthetase.

Table 4: Inhibition of the ATP synthesis driven by a proton gradient generated by bacteriorhodopsin at two different periods of illumination.

	% Inhibition	
	1 min	5 min
Oligomycin	48.0	23.6
DCCD	75.2	15.1
FCCP	49.5	20.0
Dark control	100.0	17.9

The experimental details are as given in Fig. 11. The liposomes were pre-incubated with the inhibitors during 10 min before illumination. Oligomycin 60 μ g/ml; DCCD 200 μ M; FCCP 50 μ M.

4.4. Valinomycin-induced K^+ -diffusion potential.

Fig. 12 shows that valinomycin, a K^+ selective ionophore, induces a transmembrane diffusion potential ($\Delta\psi$) that can be monitored with the potential sensitive dye Oxonol VI. This dye is only sensitive to positive membrane potentials (i.e. positive inside; Scherman and Henry, 1980; van Walraven et al., 1984). When valinomycin is added to asolectin liposomes a very rapid change in absorbance is observed, indicating an interaction between the dye and valinomycin as observed previously (Waggoner, 1976). Also it must be noticed that upon filtration of the liposomes through the column, the internal K^+ concentration was reduced due to a re-equilibration of KCl across the membrane. Stepwise additions of KCl to the medium change the absorbance in the opposite direction permitting a correlation between the added external K^+ concentration and the changes in absorbance of the dye as shown by the calibration curve in Fig. 12. The linear relationship observed

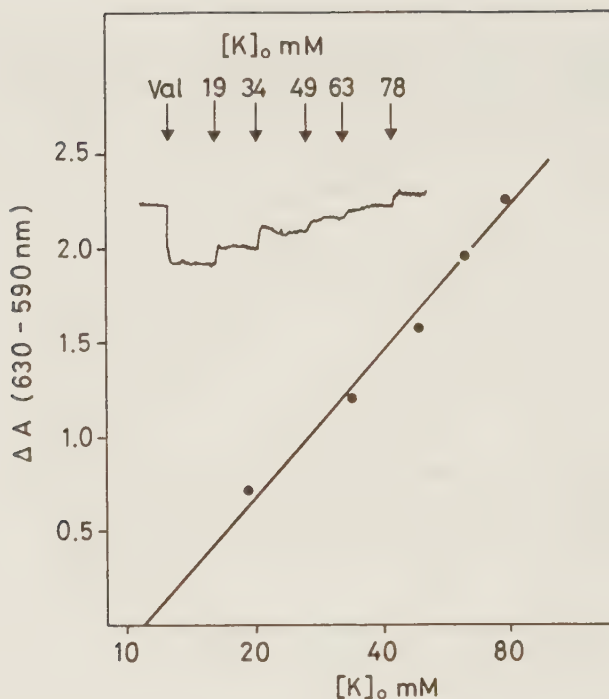


Fig. 12: Calibration curve with Oxonol VI.

Asolectin liposomes were prepared as described under Materials and Methods. The change of absorbance of Oxonol VI was monitored using a home-made dual wavelength spectrophotometer setting the monochromators at 630 (reference) and 590 nm. The buffer solutions inside and outside of the liposomes were exactly the same as those used for the experiments of ATP synthesis. After the addition of Oxonol VI to the cuvette (5.3 μ M) the signal was adjusted to 0 ΔA and then valinomycin was added (2 μ M). Aliquots of 10 μ l of a 3 M KCl solution were added giving the final concentration indicated in the figure. In a similar experiment LiCl was added instead KCl and no change was observed indicating the specificity of valinomycin for K^+ .

indicates that the absorbance change is proportional to the diffusion potential generated across the membrane and obeys the Nernst's equation. In order to calculate the $\Delta\psi$ using this equation the internal K^+ concentration should be determined. This can be done by the extrapolation as the K^+ concentration at which the diffusion potential is zero upon addition of gramicidin, an ionophore which collapses all the ionic gradients (Meunier, 1984). In the present study the absorbance signal in the presence of gramicidin was highly variable and therefore no accurate estimations of the internal K^+ levels could be made. Nevertheless the observed proportionality gives an adequate estimation of the membrane potential as a function of the external K^+ .

4.5. ATP synthesis driven by a transmembrane diffusion potential.

Since the protonmotive force ($\Delta\tilde{\mu}_H^+$) necessary to support the synthesis of ATP is composed of the sum of $Z\Delta pH$ and the membrane potential ($\Delta\psi$), it must be theoretically possible to synthesise ATP driven exclusively by a transmembrane diffusion potential (Mitchell, 1979). Using the ATP synthetase reconstituted in liposomes under the same conditions described for Fig. 11, the net ATP synthesis was measured in the presence of valinomycin and increasing external concentrations of KCl. Fig. 13 shows that the initial rates of ATP synthesis became faster as the K^+ concentration was increased in the external solution: between 5 and 104 mM the ATP synthesis catalysed by the ATP synthetase was increased by a factor of 8. The effect is better demonstrated in the inset of the same figure, where also can be seen that the rate of ATP synthesis measured under these conditions is a nonlinear function of the membrane potential. It should be noted that initially the experiment was done in the absence of any imposed pH gradient and therefore the effect observed is purely related to the diffusion potential induced by valinomycin.

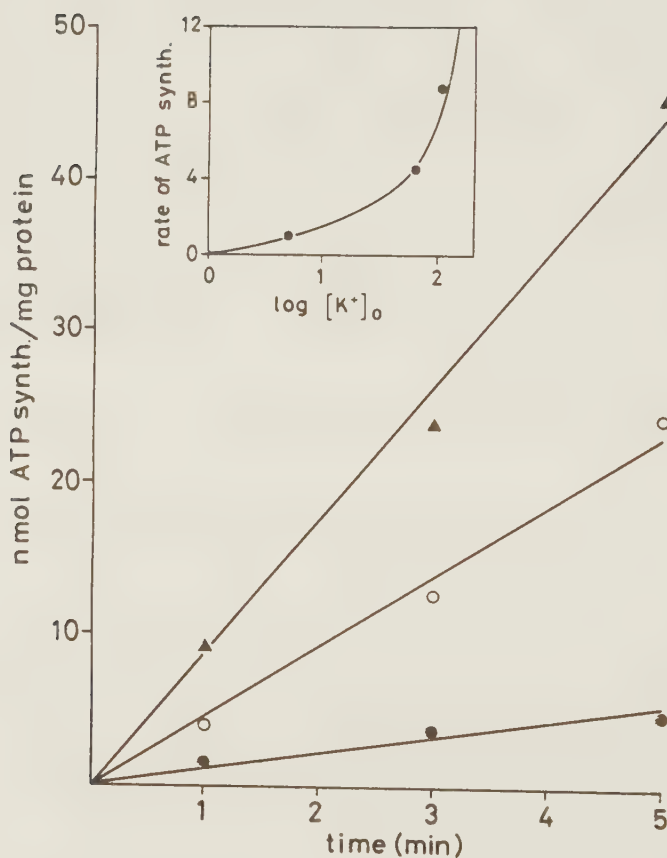


Fig. 13: Synthesis of ATP induced by a diffusion potential. The preparation of the ATP synthetase liposomes and the experimental conditions are described in Materials and Methods. The final external concentration of K^+ was 5 mM (●), 58 mM (○) and 104 mM (▲). The protein concentration was 80 μ g. The rate of synthesis is expressed as nmol/min/mg protein.

In order to abolish the diffusion potential, experiments were done in parallel in the presence of gramicidin, an unspecific channel-forming ionophore (Gómez-Puyou and Gómez-Lojero, 1977). Table 5 shows that in fact the $\Delta\psi$ -driven synthesis of ATP was inhibited up to 14, 27 and 26% at 5, 58 and 104 mM external K^+ respectively, and therefore this result verifies that the synthesis of ATP observed was certainly linked to the membrane potential.

Table 5: Effect of gramicidin on the ATP synthesis induced by a diffusion potential.

[K] _o (mM)	rate of ATP synthesis	
	Control	+ Gramicidin
5	1.10	0.95
58	4.60	3.35
104	8.80	6.55

The experimental conditions are the same as in Fig. 13. The values are in nmol/min/mg protein. Gramicidin concentration was 5 µg/ml.

On the other hand a control experiment was done in order to unequivocally prove that the measured synthesis of ATP does require an electrochemical gradient across the membrane and is not a product of the adenylate kinase, an enzyme which may synthesise ATP and AMP using two molecules of ADP. When the ATP synthetase was not reconstituted in liposomes but incubated with ADP under otherwise the same experimental conditions as those described for Fig. 13, the ATP detected over a period of 10 min was never higher than the background signal. Although some adenylate kinase activity was detected in the

ATP synthetase preparation (1% of the ATPase activity), the present control experiment proves that this enzyme was not responsible for the observed synthesis of ATP.

5. Proton conducting properties of the F_o .

The proton translocation activity of an NaBr extract (crude F_o) was assayed after reconstitution of this extract into asolectin liposomes by the dialysis method of Schneider and Altendorf (1982). A pH electrode was used to monitor the proton movements in the K^+ -loaded liposomes after addition of valinomycin. In Fig. 14 trace (A) is shown that blank liposomes do not take up protons after addition of valinomycin. As pointed out in Chapter 3.2. in the absence of external K^+ valinomycin increases the K^+ permeability of the KCl loaded liposomes generating a diffusion potential. This acts as driving force for the proton movements through the membrane. In the case of the liposomes reconstituted with the crude F_o (trace B), a rapid alcalinization of the external medium was observed after addition of valinomycin indicating that protons moved into the liposomes, and upon addition of FCCP the effect is reversed or the release of protons is accelerated. Incubation of the liposomes with DCCD ($20\mu M$) (trace C) blocks the movement of protons stimulated by valinomycin as expected.

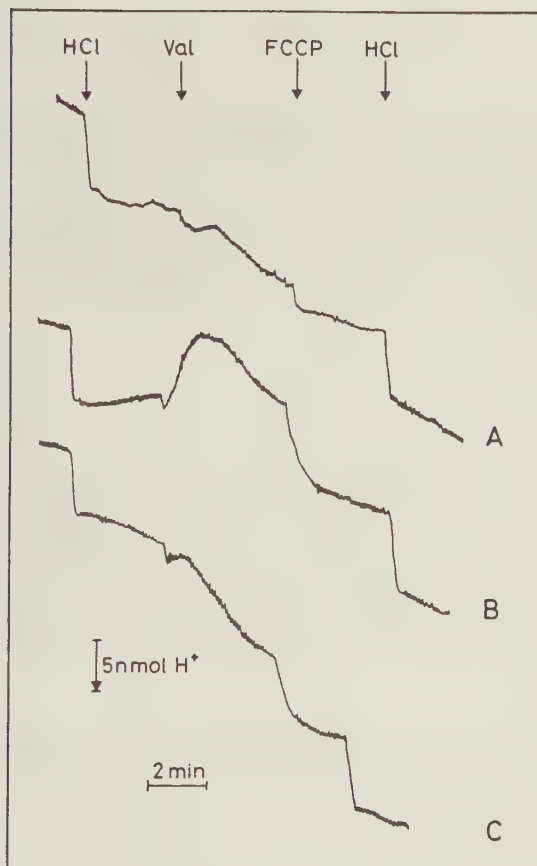


Fig. 14: Proton uptake in liposomes containing F_o.

The liposomes were prepared as described in Materials and Methods. The crude F_o was added to a concentration of 25 μ g in 2 ml final volume. The proton uptake was started by addition of valinomycin (2 μ g). (A) Asolectin liposomes, (B) F_o-liposomes, (C) F_o-liposomes preincubated with DCCD (20 μ M) for 10 min. In order to calibrate the signal, 5 μ l of 0.001N HCl were added at the beginning and at the end of the experiment.

DISCUSSION

In the present study I have purified and reconstituted in liposomes the ATP synthetase from yeast mitochondria. The enzyme incorporated in liposomes was fully functional. It was found capable of proton translocation coupled to the hydrolysis of ATP and when an electrochemical gradient was artificially imposed in the liposomes, they were able to synthesize ATP.

The right assembly and purification of the ATP synthetase.

At the initial stage of this work it was noted that controlled growing conditions for the yeast cells were required in order to have a well assembled enzyme. Two criteria were basically used to establish the right assembly of the enzyme in the inner mitochondrial membrane: a well coupled respiration (i.e. stimulation of oxygen consumption by ADP) and its sensitivity to oligomycin.

Previous works on glucose repression (more than 1% glucose in the culture medium) have shown that the mitochondria of yeast cells growing under these conditions are not properly assembled, and many of the respiratory chain proteins are present in very small amounts (Perlman and Mahler, 1974). It was also found that the F_1 and OSCP of the ATP synthetase were present but apparently not well assembled on the membrane of the mitochondria since the sensitivity to oligomycin was reduced (Tzagoloff, 1969; 1971). Only after changing the yeast cells to de-repressing conditions (glucose concentration below 1% or substrates as lactate or ethanol), did the mitochondria show the normal activities of oxidative phosphorylation.

In order to obtain good mitochondria (following the above mentioned criteria) instead of continued work with the com-

mercial yeast (i.e. grown with high concentrations of glucose), a pure culture of yeast cells was grown under aerobic conditions using ethanol (2%) as the carbon source. The mitochondria obtained from these yeast cells gave high ADP/O ratios (1.7) and the stimulation of respiration by ADP was completely inhibited by oligomycin. This result indicated that the enzyme contained in these mitochondria was a fully functional ATP synthetase and no doubts remain about its correct assembly. Therefore these yeast cells were the best starting material for the purification of a functional ATP synthetase.

Many reports have been published on the preparation of a purified ATP synthetase from many sources, including yeast mitochondria (Rott and Nelson, 1981; Ryrie and Gallagher, 1979; Todd et al., 1980; Tzagoloff, 1971). Initially some of these methods were used for the purification of the ATP synthetase in this study, but the specific activity for ATP hydrolysis was relatively low compared with the reported activities. In addition the enzyme was not very sensitive to specific inhibitors (i.e. oligomycin and DCCD). Using a modified method reported for the purification of the pig heart mitochondrial ATP synthetase (Penin et al., 1982) with α -lysolecithin as detergent, the specific activity of the purified ATP synthetase for ATP hydrolysis was improved (mean value 17 μ mol/min/mg protein). This preparation also exhibited a high sensitivity to the inhibitors oligomycin and DCCD which have been used as indicators of the correct assembly of this enzyme (Nicholls, 1982).

Although some activities of the electron transport chain of the mitochondrial membrane were found (cytochrome c oxidase and NADH cytochrome c reductase) they are in relatively small amounts (4% and 1% respectively). This was also found by Penin et al. (1982) in the pig heart complex as well as by Hughes et al. (1982), Serrano et al. (1976) and Stiggall

et al. (1978) in the bovine heart complex. Only in the case of E. coli (Foster and Fillingame, 1982), the thermophilic bacterium PS3 (Sone et al., 1975) and chloroplasts (Pick and Racker, 1979) an ATP synthetase was obtained with 8-9 subunits. However, in these instances, the enzyme complex seems to be relatively simple in comparison with the mitochondrial ATP synthetase. It has been shown that the ϵ -subunit of chloroplasts resembles the natural inhibitor protein of the mitochondrial enzyme (Nelson et al., 1972) and that the ϵ and δ subunits of E. coli are related to the δ and OSCP subunits of the mitochondrial ATP synthetase respectively (Walker et al., 1982).

Attempts were made to remove small contaminants found in the ATP synthetase preparation but this resulted in only loss of the ATPase specific activity together with the sensitivity to inhibitors, without concomitant modification of the polyacrylamide gel patterns. It seems that due to the high hydrophobicity of the subunits composing the F_0 portion of the ATP synthetase and its close relation with the other highly hydrophobic proteins of the respiratory chain that the purification of a functional complex from mitochondria has proved a particularly difficult task (Montecucco et al., 1983).

Using purified F_1 as standard the ATP synthetase preparation obtained in the present work contained all the components of the F_1 and some of the other subunits attributable to the F_0 . Although some reports identify at least 18 different subunits in the mitochondrial ATP synthetase (Ludwig et al., 1980), using a complex of only 10 subunits, some of the activities (i.e. ATPase, $^{32}P_i$ -exchange, proton translocation induced by ATP) were reconstituted (McEnery et al., 1984). Further studies are needed to conclude whether it is possible or not to have a mitochondrial ATP synthetase with a minimal subunit composition and capable of all its functions when re-inserted in a membrane.

The F_1 portion of the ATP synthetase is well characterized due in part to its relatively easy purification and its enzymatic activity and therefore its subunits composition and stoichiometry are well known. In the case of the F_0 , only in very simple systems such as bacteria and chloroplasts (Schneider and Altendorf, 1982; Okamoto et al., 1977; Nelson et al., 1980) is the subunit composition known. In the case of the mitochondrial F_0 there are some reports of its purification (Alfonzo et al., 1981; Galante et al., 1981; Glaser et al., 1980; Tzagoloff et al., 1968) but the exact subunit composition is still not clear because one of the criteria most widely used for identification of the different polypeptides composing the ATP synthetase is gel electrophoresis, and the resolution of some of the methods used is not high enough (Ludwig et al., 1980).

Other criteria used are the capacity to reconstitute the sensitivity to oligomycin of the ATPase activity and ATP- P_i exchange in the presence of F_1 (Galante et al., 1981; Tzagoloff et al., 1968) and more recently the ability of proton translocation in liposomes containing the F_0 by the imposition of a ΔpH and a K^+ -diffusion potential or upon addition of ATP. At present the proton translocating ability has been mainly assayed in the bacterial systems (Negrin et al., 1980; Okamoto et al., 1977; Schneider and Altendorf, 1982). In bacterial systems it was also shown that not all the subunits of the F_0 were necessary to reconstitute the proton translocating activity (Sone et al., 1978). It is possible that the discrepancy of subunits composition of the F_0 between the different preparations (6 to 10 subunits) is due to the methodology employed to check the requirements to reconstitute a full functional ATP synthetase (capable of net ATP synthesis). Conceivably due to the complexity of the inner mitochondrial membrane, some of the subunits may have regulatory roles in conjunction with the components of the respiratory chain.

The purified and reconstituted ATP synthetase is indistinguishable from the native enzyme.

The study of purified membrane proteins requires that after their solubilisation with detergents or chaotropic agents they must be re-incorporated in artificial membranes in order to reconstitute function. In some cases it is enough to provide the protein with a membrane support (Knowles et al., 1976), but in other cases it is necessary to incorporate the protein in closed membrane vesicles (i.e. liposomes) in order to be able to reconstitute the vectorial activity (Racker et al., 1979; Eytan, 1982). The reconstitution procedures are very variable and one of the most widely used is cholate dialysis (Kagawa and Racker, 1971). However, this procedure in some cases requires a prolonged dialysis (i.e. several days), and the activity of the enzyme may be modified. Other procedures involve sonication of the protein with a suspension of phospholipids (Racker, 1973) but many proteins are damaged by the strong sonic irradiation. A very effective method was introduced by Kasahara and Hinkel (1976) in which the protein is reconstituted in pre-formed liposomes by freeze-thaw cycles followed by a very short period of sonication. This last method was used to reconstitute the ATP synthetase in this study. The cholate dialysis was also tested but due probably to the long dialyzing time (18 hr) the ATP synthetase was somewhat inactivated.

In morphological analysis of the reconstituted ATP synthetase in asolectin-liposomes by means of electron-microscopy it was found that the liposomes were relatively uniform in size (100-200 nm) and using the freeze-etching method, the surface of the liposomes could be observed. Only in the case of liposomes reconstituted with the ATP synthetase, were particles of about 90 Å found, which corresponds to the reported size of the F_1 portion in the ATP synthetase (Fernández-Morán et

al., 1964; Soper et al., 1979). In submitochondrial particles, the size of the proteins oriented towards the outside was the same, and it is known that in submitochondrial particles the F_1 has an inside-out orientation with respect to the mitochondria. Therefore the method used for reconstitution of the ATP synthetase in liposomes seems to orientate preferentially the ATP synthetase with the hydrophilic moiety exposed to the external medium, allowing access of the substrates (ATP or ADP- P_i) to the enzyme.

Steady-state kinetics of the hydrolytic activity of the ATP synthetase and the free F_1 confirms the reported negative cooperativity for Mg-ATP (Gresser et al., 1982; Wieker and Hess, in press), indicating that the enzyme has all its catalytic sites still intact. Thus, no modifications due to the purification procedure and the reconstitution in liposomes occurred. Other experiments that confirm the integrity of the ATP synthetase were those involving proton translocation induced by ATP hydrolysis. In this case, the enzyme reconstituted in liposomes was capable during the hydrolysis of ATP to work in the reverse direction pumping protons from the outside to the interior of the liposomes. This activity was inhibited by energy-transfer inhibitors (oligomycin and DCCD) as well as by the uncoupler nigericin. Therefore the ATP synthetase purified and reconstituted in liposomes, has the same properties of the enzyme in its natural environment, permitting detailed studies aimed at gaining more information about its molecular mechanism.

Net ATP synthesis linked to electrochemical gradients.

In order to make quantitative studies on the synthetic activity of the ATP synthetase reverse phase HPLC was employed for the measurement of the ATP synthesised. This technique has some advantages over the methods most common employed.

The method is highly sensitive (down to 10 pmoles), the preparation of the samples for the quantification does not require many manipulations (e.g. reduction in sample loss), and it is the nucleotide itself that is measured. Most of the other methods are indirect, and have the same problem that this new method has, which is the hydrolysis of the ATP newly synthesised by ATPase activity or spontaneous degradation. The only method which can prevent this effect is that using a coupled reaction with hexokinase and glucose, but the subsequent determination is not as sensitive as the one reported here and the calculations require many assumptions (Aflalo and Shavit, 1982). The other method using $^{32}\text{P}_i$ requires many extractions steps, a good determination of the specific activity and can measure unspecifically the synthesis of phosphate products from other reactions as ATP- $^{32}\text{P}_i$ exchange. The luciferin-luciferase method is also very sensitive (pmoles range), the reaction is specific for ATP but is also very sensitive to other substances that can be present in the sample (CL^- , high amounts of TCA, etc.) making it very unstable and the internal standard that should be included in every determination is not as reproducible as in the HPLC method. Thus, the new method developed using HPLC for ATP determination was considered the most reliable.

The overall reaction in oxidative and photophosphorylation consists of two steps, formation of a $\Delta/\tilde{\mu}_H$ by electron transfer reactions and synthesis of ATP utilizing the electrochemical potential of protons (Fillingame, 1980; Futai and Kanazawa, 1983; Nicholls, 1982). The ATP synthetase is the enzyme responsible for ATP synthesis in the energy-transducing membranes utilizing the energy stored in the electrochemical potential. It is also known that this enzyme can generate a $\Delta/\tilde{\mu}_H$ comparable to that produced by the electron transfer mechanisms, when the ATP synthetase works in the reverse direction (ATP hydrolysis) (Mitchell and Moyle, 1968).

The first demonstration of an artificially generated $\Delta/\tilde{u}_H^+$ capable of driving ATP synthesis came from isolated chloroplasts subjected to a ΔpH of 4 units across the membrane (Jagendorf and Uribe, 1966). In mitochondria, ATP synthesis was induced by the imposition of a $\Delta/\tilde{u}_H^+$ generated by its two components: ΔpH and a K^+ -diffusion potential across the membrane of submitochondrial particles (Thayer and Hinkle, 1975). The experiments done with intact organelles show that ATP can be synthesised using $\Delta/\tilde{u}_H^+$ as a unique energy input, but they did not show if the electrochemical potential generated by the electron-transfer chain is directly linked to ATP synthesis or to some other mechanisms of proton pumping on a side-path which are in equilibrium with an alternative intermediate (Fillingame, 1980). Therefore it has been necessary to purify and reconstitute the ATP synthetase in artificial membranes in order to study the coupling between electrochemical gradients and the synthesis of ATP.

Certainly it has been found that the ATP synthetase from different organisms are able to synthesise ATP when a ΔpH was applied to the reconstituted ATP synthetase in the presence of K^+ and valinomycin (Kagawa, 1978; Pick and Racker, 1979; Ryrle et al., 1979) or when an electric field was applied in voltage pulse experiments (Gräber et al., 1982; Knox and Tsong, 1984; Rögner et al., 1979). Therefore the possibility of an alternative intermediate can be discarded since the above mentioned experiments were done with the ATP synthetase complex being virtually the sole protein component present.

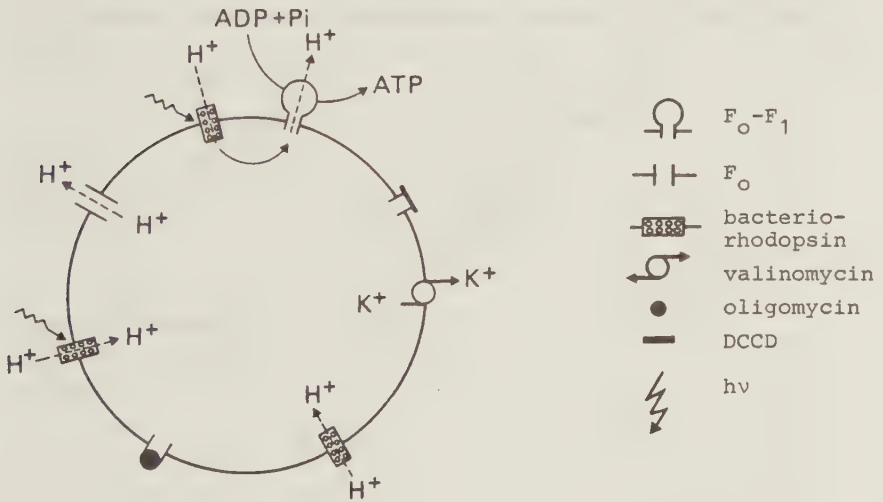
It is important to note (as pointed out by Maloney, 1982) that in the different energy-transducing membranes the synthesis of ATP can take place depending on the general properties of the system. Thus in mitochondria, where the buffering capacity is high on either side of a membrane which is almost impermeable to all ions, extrusion of protons

charges the membrane capacitance without appreciably modifying the pH. In this case the membrane potential alone may drive the synthesis of ATP. On the other hand chloroplasts are permeable to ions (except H^+ and OH^-) and their buffering capacity is low. When protons are pumped to the interior, the charge moved as H^+ is compensated by other chemical species moving passively, affecting the chemical gradient. In this case the ΔpH is the factor which determines ATP synthesis. Bacteria may use either type of energy depending upon the conditions of the medium. All these possibilities can be better studied in reconstituted systems in which the electrical and chemical gradients are controlled in order to study their direct role on the catalytic activities of the ATP synthetase.

In the present study net synthesis of ATP induced by an artificial $\Delta\tilde{u}_H^+$ was analysed, using two different methods for the generation of the electrochemical potential: a) Bacteriorhodopsin was co-reconstituted with the ATP synthetase in order to generate a ΔpH . The use of bacteriorhodopsin for this kind of experiments has many advantages. Under defined conditions it can pump protons from the external medium to the interior of the liposomes. Thus resembling the activity of the electron-transport chain, and the generation of the ΔpH does not involve such drastic conditions as the pH-jump experiments. Racker and Stoeckenius (1974) have shown ATP synthesis upon illumination of liposomes containing bacteriorhodopsin and bovine heart ATP synthetase.

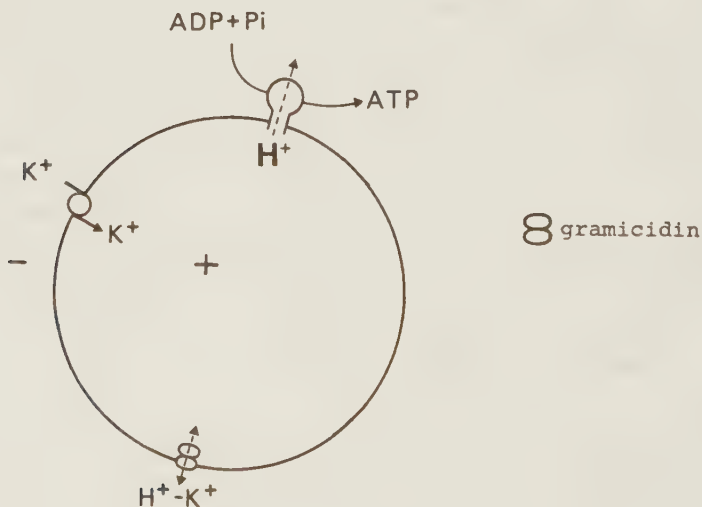
b) A valinomycin-induced K^+ -diffusion potential was generated in liposomes containing only the ATP synthetase. Recently generation of a membrane potential was achieved by subjecting membrane preparations containing the ATP synthetase to electric field pulses (chloroplasts, submitochondrial particles or reconstituted liposomes), (Knox and Tsong, 1984; Rögner et al., 1979; Schlodder et al., 1982). Under these conditions

ATP is synthesised, but there are still some problems in the interpretation and the calculations of the rate of synthesis observed, due to the polarization of the vesicles during the experiments (i.e. one half of the vesicles have an inverted polarization with respect to the other). A uniform and well controlled membrane potential is achieved using the K^+ -selective ionophore valinomycin. Preliminary reports indicate that also under these conditions ATP can be synthesised in liposomes containing the ATP synthetase (Dinant and Kaminski, 1984; van Walraven et al., 1984).



scheme I

The schemes I and II help to clarify the results obtained in the present work: In one case (scheme I) upon illumination, bacteriorhodopsin generates a ΔpH (accelerated by the presence of valinomycin) capable of inducing ATP synthesis as was originally postulated (Mitchell, 1979). In the second case (scheme II) the generation of an electrical gradient is able to effect ATP synthesis. When the membrane potential is generated by changing specifically the membrane permeability to K^+ , protons are forced to leave the liposomes down the electric field. In this way the protons flow outwards through the ATP synthetase forming ATP. The synthesis of ATP is diminished in the presence of gramicidin, which can form unspecific channels by increasing the membrane permeability to K^+ and protons, dissipating the electrochemical gradient (scheme II).



scheme II

Therefore out of these observation can be concluded that the electrical membrane potential is the unique driving force for the ATP synthesis.

The effects of energy transfer inhibitors and uncouplers on synthesis of ATP can be also clarified. It was found that the inhibitory activity of these agents on the synthesis of ATP was diminished as a function of time (see Table 4). Nevertheless the hydrolytic activity was still more than 90% inhibited by oligomycin and DCCD and as it was expected no effect on the ATPase activity by FCCP was found. These findings can be explained as follows (scheme I): it is possible that in the reconstituted liposomes some F_o lack the F_1 and in the presence of the inhibitors which presumably act on the F_o , the liposomes are better coupled because the inhibitors can close the open F_o and protons cannot diffuse out of the liposomes. Therefore the liposomes are tighter and the efficiency of the electrochemical potential is improved. For example in submitochondrial particles, oligomycin has been used to restore the oxidative phosphorylation (Lam et al., 1967; Matsuno-Yagi and Hatefi, 1984). An alternative explanation might invoke a change in the affinity of the enzyme to these agents due to a conformational transition during the two processes (synthesis and hydrolysis) as was found for DCCD in chloroplasts and submitochondrial particles (Gräber et al., 1982; Hammes, 1982; Matsumo-Yagi and Hatefy, 1984). The effect of these inhibitors during the catalytic cycle of the ATP synthetase is not well understood but more detailed kinetic studies on their mode of action may provide a better understanding of the molecular mechanisms of the ATP synthetase.

The fact that with FCCP also a time-dependent decrease of the inhibition of the ATP synthesis was observed (Table 4) may have also interesting implications: Under the most strict

premises of the chemiosmotic theory, FCCP must inhibit the ATP synthesis by abolishing the ΔpH . This apparent discrepancy can however, be explained considering the "electrodeic view" of electrochemical proton gradients associated with energy-transducing membranes proposed by Kell (1979). According to his interpretation, there are diffusion barriers to protons near the surface of energy-transducing membranes separated from the bulk phase. The presence of these barriers permits the protons moving across the membrane to be immediately used by the ATP synthetase without been lost in the bulk phase. Therefore FCCP may modify the bulk distribution of protons without affecting sensibly the local movements of protons close to the membrane. A similar explanation was proposed in experiments with whole cells by Michel and Oesterhelt (1980). In the dark control the diminished inhibition after five minutes incubation, cannot be easily explained and further experimental evidence is required. As pointed out previously the adenylate kinase (a soluble enzyme) can not be responsible of this apparent discrepancy, since the non-reconstituted preparation under otherwise similar conditions, was incapable of forming ATP.

Concluding remarks.

It was found that the protein:lipid ratio of the inner mitochondrial membrane is of the order of 4:1 (Sjöstrand and Barajas, 1970). This is extremely high when compared with other biological membranes, making the isolation of particular transmembrane proteins a difficult problem. This applies specially to the purification of the F_0 portion of the ATP synthetase which by itself lacks an enzymatic activity and may be tightly bound to other hydrophobic proteins. Nevertheless as demonstrated in this work, a complete and fully functional mitochondrial ATP synthetase ($\text{F}_0\text{-F}_1$ complex) was isolated under conditions in which a mild and relatively specific detergent was used.

It must be emphasized that the isolated ATP synthetase reconstituted in liposomes catalyses the net synthesis of ATP linked to electrochemical gradients. Returning from the reconstituted system to the native one, it is particularly interesting that an electrical membrane potential can apparently drive the synthesis of ATP in the absence of a preformed ΔpH across the liposomal membrane. A similar effect was observed several years ago with isolated mitochondria but, due to the complexity of the system, it was not properly interpreted (Cockrell et al., 1967). In that work it was concluded that a K^+ pump working in reverse was responsible for the ATP synthesis observed. To date such a pump has not been isolated from mitochondria. On the other hand as has been pointed out previously and supporting the present findings, due to the high buffer capacity of the mitochondria (Maloney, 1982; Mitchell and Moyle, 1968), the oxidative phosphorylation in these organelles appears to be driven or regulated by the membrane potential.

Since the same behaviour is observed with the isolated ATP synthetase, this gives emphasis to the possible importance of the membrane potential as a regulator of this enzyme. This is reinforced by the dependence of ATP synthesis as a function of membrane potential (Fig. 13), which indicates that within a narrow range of membrane potentials the rate of formation of ATP increases exponentially. A very similar relationship has been observed with submitochondrial particles and isolated chloroplasts (Knox and Tsong, 1984; Schlodder et al., 1982).

Two alternative mechanisms can explain this observation. The generated diffusion potential acts as the direct driving force for ATP synthesis. The vectorial efflux of protons through the ATP synthetase is coupled to the ATP synthesis (scheme II). Concomitantly the F_o may be a kind of voltage-

gated H^+ -channel as is already very well known for other cationic channels (Keynes, 1983), but this remains to be tested (Schindler and Nelson, 1982). Alternatively, the present stage of this work does not rule out the possibility that a voltage-dependent conformational change (Gräber et al., 1982) releases tightly bound ATP to the medium (Boyer et al., 1982), giving an apparent increase in the rate of ATP formed.

Having developed adequate and simple experimental models, as well as sensitive methods to monitor the catalytic activities of the ATP synthetase, the future research in this field will demand a better characterization of the intra-membranal part of the ATP synthetase from mitochondria (e.g. the minimal structural requirements of a functional F_o). Additionally a separate reconstitution of the different subunits which compose the full complex (Yoshida, et al. 1977) should provide the molecular basis for understanding the function and regulation of the ATP synthetase.

SUMMARY

I have studied the functional properties of the isolated ATP synthetase (F_0 - F_1 complex) of yeast mitochondria reconstituted in liposomes, in order to analyze quantitatively the oxidative phosphorylation of ADP using the principles of the chemiosmotic theory (Mitchell, 1979).

The ATP synthetase was solubilized with α -lysolecithin, and using a freeze-thaw method reconstituted in asolectin liposomes. The following results were obtained:

1) The isolated ATP synthetase is composed of several different polypeptides, 12 of them could be identified with the reported components of the complex.

2) Freeze-etching electron microscopy of the reconstituted liposomes and submitochondrial vesicles show characteristic membrane particles on their surface with a diameter of 90 Å. These presumably represent the F_1 part of the ATP synthetase. Such particles were not observed in blank liposomes.

3) The ATP hydrolysis was inhibited by oligomycin and DCCD almost completely (> 90%) and not affected by the ionophore nigericin, indicating the right assembly of the enzyme. On the other hand the ATP synthetase reconstituted in liposomes and the purified free F_1 gave very similar steady state kinetic constants for ATP hydrolysis, suggesting that the hydrolytic activity of the F_0 - F_1 complex is independent of its membrane-bound state.

4) The proton translocating activity coupled to ATP hydrolysis was assayed using the fluorescent probe ACMA. As expected this activity was inhibited by oligomycin, DCCD and the ionophore nigericin.

5) The F_0 reconstituted in liposomes was also capable of a passive proton translocation upon generation of a diffusion potential. This activity was inhibited by DCCD.

6) The net ATP synthesis was directly measured by means of reverse phase HPLC, under two different conditions:

a) Bacteriorhodopsin was co-reconstituted in liposomes with the ATP synthetase in order to generate a light-driven ΔpH . The rate of ATP synthesis was dependent on the illumination time of the liposomes and was of the order of 19 nmol/min/mg protein. b) In the absence of an artificially pre-formed ΔpH and using valinomycin, a K^+ -specific ionophore, a diffusion potential was generated, the rate of ATP synthesis was of the order of 8.8 nmol/min/mg protein (when the external K^+ was 104 mM).

Out of these observations I can conclude:

1) A direct and sensitive method suitable for a quantitative analysis of ATP synthesis has been developed.

2) The solubilized and purified ATP synthetase can be reconstituted into liposomes with retention of its catalytic activities. According to the chemiosmotic theory the reconstituted enzyme is able to translocate protons through the membrane and to use the electrochemical gradient of protons to synthesise ATP.

3) The membrane potential gives enough driving-force to synthesise ATP. Additionally it is suggested that the membrane potential has an important and effective regulatory role on the ATP synthetase activity.

4) This study provides a well controlled experimental system for further investigations on the molecular mechanism of the mitochondrial ATP synthetase.

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